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1 Your reference
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2 Patent application number
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3 Full name, address and postcode of the or of each applicant *(underline all surnames)*
Dr Camilo Colaco
107 Foster Road
Cambridge CB2 2JN
United Kingdom

Patents ADP number *(if you know it)*
6814107001

If the applicant is a corporate body, give the country/state of its incorporation

4 Title of the invention
Vaccines from infectious agents

5 Name of your agent *(if you have one)*
Dr Camilo Colaco

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*
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Description 6

Claim(s) 1 (5 claims)

Abstract 1

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Dr Camilo Colaco Tel 01223 841870

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TITLE: VACCINES FRO[redacted]NFECTIOUS AGENTS

The present invention relates to a vaccine and a method for producing a vaccine. More specifically, it relates to methods of producing vaccines of stress-induced proteins from pathogenic organisms and the compositions obtained thereby.

BACKGROUND OF THE INVENTION

An important component of any human immune response is the presentation of antigens to T cells by antigen presenting cells (APCs) such as macrophages, B cells or dendritic cells. Fragments of foreign antigens are presented on the surface of the macrophage in combination with Major histocompatibility complex (MHC) molecules, in association with helper molecules, such as CD4 and CD8 molecules. Such "antigenic fragments", presented in this way, are recognised by the T cell receptor of T cells, and the interaction of the antigen with the T cell receptor results in antigen-specific T cell proliferation, and secretion of lymphokines by the T-cells. The nature of the antigenic fragment presented by the APCs is critical in establishing immunity.

Heat shock proteins (HSPs) form a family of highly conserved proteins that are widely distributed throughout the plant and animal kingdoms. On the basis of their molecular weights, HSPs are grouped into six different families: small (hsp 20-30kDa); hsp40; hsp60; hsp70; hsp90; and hsp100. Although HSPs were originally identified in cells subjected to heat stress, they have been found to be associated with many other forms of stress such as infections, and are thus more commonly known as "stress proteins" (SPs).

Members of the mammalian hsp90 family include cytosolic hsp90 (hsp83) and the endoplasmic reticulum counterparts hsp90 (hsp83), hsp87, Grp94 (Erp99) and gp97. See for instance, Gething et al. (1992) *Nature* 355:33-45. Members of the hsp70 family include cytosolic hsp70 (p73) and hsp70 (p72), the endoplasmic reticulum counterpart BiP (Grp78), and the mitochondrial counterpart hsp70 (Grp75). Members of the mammalian hsp60 family have only been identified in the mitochondria. The latter family of HSPs is also found in procaryotes which also contain three other major families of HSPs, the GroEL, GroES, DnaJ and DnaK families. As in eucaryotes, the procaryotic HSPs are also thought to function in the folding of nascent polypeptide chains during protein synthesis.

In eucaryotic cells which have intracellular membrane organelles, one of the roles of HSPs is to chaperone peptides from one cellular compartment to another and to present peptides to the MHC molecules for cell surface presentation to the immune system. In the case of diseased cells, HSPs also chaperone viral or tumour-associated peptides to the cell-surface. Li and Sirivastava (1994) *Behring Inst. Mitt*, 94: 37-47 and Suzue et al. (1997) *Proc.Natl.Acad.Sci. USA* 94:

13146-51. The chaperone function is accomplished through the formation of complexes between HSPs and proteins and between HSPs and viral or tumour-associated peptides in an ATP-dependent reaction. HSPs bind a wide spectrum of peptides in an ATP dependent manner. The bound peptides appear to be a random mix of peptides. The mixtures and exact natures of the peptides have not been determined. The association of HSPs with various peptides has been observed in normal tissues as well and is not a tumour-specific phenomenon. See Srivastava (1994) *Experimentia* 50: 1054-60.

In a therapeutic context, it has been proposed to use mammalian HSPs as vaccines. WO 97/10000 and WO 97/10001 disclose that a mixture of heat shock proteins (HSPs) isolated from cancer cells or virally infected cells are capable eliciting protective immunity or cytotoxic T lymphocytes to the cognate tumour or viral antigen. However, in contrast, HSPs isolated from normal cells are unable to elicit such immunity. It is now thought that HSPs are not immunogenic *per se*, but are able to elicit immunity because of their association with tumour or virus specific antigenic peptides that are generated during antigen processing. Specifically, the peptides associated with the HSPs are immunogenic, and are presented to the T cells. HSPs stripped of associated peptides lose their immunogenicity (Udono, H. and Srivastava, P. K., Journal of Experimental Medicine, *178*, page 1391 *ff*, 1993). To date, the nature of these peptides has not been determined.

It is currently believed that the antigenicity of HSPs results not from the HSP per se, but from the complex of peptide associated with the HSP. This conclusion is based on a number of characteristics of the complexes. There are no differences in the structure of SPs derived from normal and tumour cells. Certain complexes lose their immunogenicity upon treatment with ATP, Udono et al. (1993) *J.Exp.Med.* 178: 1391-96. Such loss of immunogenicity is due to dissociation of the complex into its HSP and peptide components. The immunogenicity of HSP preparations depends upon the presence of phagocytic cells, such as macrophages and other APCs. It is now thought that HSPs are taken up by macrophages, and those peptides associated with the HSPs are then presented by MHC class I molecules of the macrophage. In this way, a T cell response is initiated.

The use of mammalian HSP-complexes from infected cells as vaccine against intracellular pathogens has been disclosed in WO 95/24923. HSPs isolated from viral infected cells have been suggested as a source of antigenic peptides, which could then be presented to T cells. This necessitates the production and purification of HSPs from such cells. The use of HSP proteins as vaccine components has further been disclosed in WO 97/10000, WO 97/10001 and WO 97/100002 which disclose that a mixture of heat shock proteins (HSPs) isolated from cancer cells

or virally infected cells a[illegible]apable eliciting protective immunity or [illegible]toxic T lymphocytes to the cognate tumour or viral antigen. HSPs isolated from tumour cells or viral infected cells have been suggested as a source of antigenic peptides, which could then be presented to T cells. This necessitates the production and purification of HSPs from such cells. Furthermore WO 98/34641 discloses that surprisingly low amounts of HSPs are required to immunise animals against tumour or viral antigens. All these HSP vaccine approaches utilise mammalian HSPs from the species for immunisation of the desired animal species.

HSPs from the extracellular pathogens themselves have also been utilised to immunise mammalian species as antigens *per se* but not as carriers of antigenic peptides except as conjugates or hybrid fusion proteins. Thus WO 95/14093 discloses that the use of Helicobacter pylori HspA and B as immunogens elicits a good antibody response against these proteins that is effective against the organism. Similarly, WO 96/40928 14093 discloses that the use of HSP 70 and 72 from Streptococcus elicits a good antibody response against these proteins that is effective against the organism. Furthermore WO 90/02564 14093 discloses that the use of Trypanosomal, Mycoplasmal or Mycobacterial HSPs, and especially HSP70, as immunogens ellicts a good antibody response against these proteins that should be effective against the respective organisms. Alternatively US 05830475 uses proteins expressed as fusions of the M.Bovis HSP genes as antigens and US 05736164 uses the T-cell epitope of hsp65 conjugated to poorly immunogenic antigens.

Surprisingly however, endogenous HSP-complexes from procaryotic and protozoan species, and more especially HSP-complexes from these organisms treated by heat shock or other stresses to increase their intracellular HSP content, have not been used as vaccines to immunise vertebrates such as mammals, birds or fish against these infectious disease pathogens.

SUMMARY OF THE INVENTION

Therefore, in a first aspect, the present invention provides a method for producing a vaccine, comprising the steps of:

a) exposing pathogenic organisms to stress inducing stimuli such as heat;

b) extracting the endogenous stress induced proteins from the treated organisms; and

c) use of the extracted proteins in the preparation of a vaccine.

It is surprising that the treatment of pathogens with stress-inducing stimuli such as heat produces HSP-complexes which are more immunogenic than the HSPs themselves or HSPs derived from uninduced organisms. The most notable aspect of immunity elicited by these induced

HSP-complexes is the exceptionally high neutralising antibody titres and the long-term memory obtained compared to that induced by immunisation by the HSPs themselves.

The term "vaccine" as used herein, refers to any composition which stimulates the immune system such that it can better respond to subsequent infections. It will be appreciated that a vaccine usually contains an immunogenic determinant and an adjuvant, which non-specifically enhances the response to that determinant. The term "pathogenic organism" as used herein, refers to any pathogen that causes a disease in a vertebrate, including bacterial, protozoal and fungal species.

The immunogenic determinant for the present invention may be delivered in combination with an adjuvant. Suitable adjuvants are readily apparent to the person skilled in the art, such as Quil A, Detox, ISCOMs or squalene. However, it will be appreciated that the vaccine of the present invention may also be effective without an adjuvant. Such a vaccine may be given by any suitable means, such as orally, or by injection.

The term "stress proteins" and "heat shock protein", as used herein, are standard in the art, and includes those proteins that comprise the GroEL, GroES and DnaK and DnaJ families of bacterial HSPs and related families in other extracellular pathogens. These families are named on the basis of the size of the peptides which they encode. The families are highly conserved between species. In addition, many bacteria also express homologues of eucaryotic proteins. Preferably the vaccine contains at least one HSP derived from the stressed pathogen. We particularly prefer that the GroEL, GroES, DnaK and DnaJ families of proteins are used as immunogenic determinants in the present invention, with DnaJ and GroEL most preferred. While the method of heat stress induction is readily available to one skilled in the art, we prefer a heat treatment of the pathogen at a temperature 5-8°C above the normal growth temperature of the organism. Without being constrained by theory, it is thought that the treatment works either to specifically induce those HSPs most able to interact with peptides, or to induce those HSPs which are most easily phagocytosed by APCs, or both. Comparative immunogenicity can be determined by *in vivo* testing on animal models. Other suitable methods will be readily apparent to the person skilled in the art, 'Current Protocols in Immunology', Wiley Interscience, 1997.

The extraction and purification of HSPs of the present invention from the extracellular pathogen is standard in the art. Suitable methods include disruption of treated organism by homogenisation or sonication, followed by centrifugation to obtain a crude HSP preparation in the supernatant. It will be appreciated that the crude endogenous HSP preparations obtained may be used as the vaccine preparation of choice. Optionally, the HSPs may be purified further by the use of ADP binding columns or other suitable methods readily available to the person skilled in the art, see for example those described in WO 97/10000 and WO 97/10001.

It will be appreciated that the present invention also includes methods of producing and isolating specific immunogenic HSPs from the extracellular pathogen, essentially as described above. HSPs derived in this way may be used to isolate samples of antigenic peptides, for example.

Methods of medical treatment using the vaccine of the present invention are also included in the present invention. Such methods include the administration of a pharmaceutically acceptable quantity of immunogenic determinant, optionally in combination with an adjuvant, sufficient to elicit an immune response in a patient.

The following examples are provided to illustrate but not limit the invention.

EXAMPLES

Example 1: Preparation of heat-induced HSPs.

One specific example of the HSP preparation process is as follows. M.Bovis was grown to stationary phase and heat-shocked at 42°C for 0.5hr or at 39°C for 5hr and cultured overnight. Cells are then washed in phosphate buffered saline (PBS). The cells are then re-suspended in homogenisation buffer. The homogenisation buffer may be a hypotonic buffer, such as 10 mM phosphate pH 7.4 with 2mM $MgCl_2$, after which the cells are then disrupted using a cell homogeniser (e.g. French press, Ultraturrax or Waring blender) or lysed by the use of detergents such as Tween or Triton, complement lysis at 37°C or by repeated freeze-thaw cycles (e.g. in liquid nitrogen). The cell lysate is then treated by centrifugation, typically 3-5000 x g for 5 minutes, to remove the nuclear and cell debris, followed by a high speed centrifugation step, typically 100,000g for 15-30 minutes.

The supernatant thus obtained may be concentrated for immediate use as, or further processed to give, a protein complex suitable for use in a vaccine. This can be done simply by ammonium sulphate precipitation which uses a 20-70% ammonium sulphate cut. Specifically, 20 % (w/w) ammonium sulphate is added at 4°C, the precipitate is discarded, followed by the addition of more ammonium sulphate to bring the concentration to 70 %. The protein precipitate is harvested by centrifugation, and then dialysed into an appropriate physiological, injectable buffer, such as saline, to remove the ammonium sulphate before use. It will be appreciated that the HSPs isolated in this way are not purified to homogeneity, but are nevertheless suitable for use as a vaccine component.

If more purified HSP preparation is required, then the HSPs may be purified from the supernatant by affinity chromatography on matrices carrying adenosine diphosphate, such as ADP-agarose or ADP-sepharose. These methods are standard in the art, and are outlined in WO 97/10000, WO 97/10001 and WO 97/10002.

HSPs may be used at any suitable concentration. We prefer that the amount of induced HSP complex that is administered is in the range of 10-600 μg, preferably 10-100 μg, most preferably 25 μg.

In order to determine the immunogenicity of stress protein-peptide complexes, T cell proliferation assays may be used. Suitable assays include the mixed-lymphocyte reaction (MLR), assayed by tritiated thymidine uptake, and cytotoxicity assays to determine the release of ^{51}Cr from target cells. Both of these assays are standard in the art (see 'Current Protocols in Immunology', Wiley Interscience, 1997). Alternatively, antibody production may be examined, using standard immunoassays or plaque-lysis assays, or assessed by interuterine protection of a foetus (see 'Current Protocols in Immunology').

Example 2: Immunisation with induced HSPs; immunity in vaccine recipient

HSPs were prepared as described in Example 1 above and mice and rabbits vaccinated with 1-10 micrograms of the stress-protein containing extract in phosphate buffered saline and boosted with identical vaccine dosages a month after the primary injection. Induction of immunity to pathogen was assayed by Western blot analysis using total M.bovis proteins. Antibody titres of 1:1-10,000 were routinely obtained and cytotoxic T-cell activity directed against pathogen infected cells could also be detected in the immunised mice. Challenge of the rabbits with fixed M.bovis at 6, 12 and 18 months periods after the initial immunisations resulted in the production of good antibody responses with titres of 1:1-10 000 indicating good memory responses in the immunised animals.

CLAIMS

1 A method for producing a vaccine, comprising the steps of:

a) exposing pathogenic organisms to stress inducing stimuli such as heat;

b) extracting the endogenous stress induced proteins from the treated organisms; and

c) using the extracted proteins in the preparation of a vaccine.

2 A method as claimed in claim 1 wherein the cellular extract consists essentially of induced heat shock proteins.

3 A method for producing a vaccine substantially as hereinbefore described in any one of the examples.

4 A vaccine obtained according to the method of either of claims 1 or 3.

5 A vaccine as claimed in claim 1, wherein the cellular extract consists essentially of induced heat shock proteins.

ABSTRACT

The present invention relates to a method for producing and isolating specific immunogenic endogenous heat shock proteins induced by the treatment of extracellular pathogens with stress inducing stimuli and vaccines prepared from such proteins.